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A new method for the encapsulation of mammalian cells

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Abstract

A new encapsulation method was developed for the cultivation of mammalian cells. The capsules were produced using a solution of sodium cellulose sulphate (CS)(1.5%) and poly-dimethyl-diallyl-ammoniumchloride (PDMDAAC). When CS droplets fell into the precipitation bath consisting of a 2% solution of PDMDAAC, immediately a membrane at the interphase was built up. The influences of varying encapsulation process parameters on capsule characteristics, cell growth, and monoclonal antibody production were tested. This new method showed advantages when compared to other methods mainly due to time simplicity of the whole process.

Abbreviations: CS: Sodium cellulose sulphate; CMC: Sodium carboxymethyl-cellulose; DMEM: Dulbecco's modified Eagle medium; FCS: fetal calf serum; mAb: Monoclonal antibodies; PBS: Phosphate buffered saline; PDMDAAC: Poly-dimethyl-diallyl-ammoniumchloride; PEI: Poly-ethylene-imine; PLL: Poly-L-lysine; aqua dest.: distilled water

Introduction

Several methods have been developed for the microencapsulation of mammalian cells alginate-PLL-PEI (or other crosslinking substances)-capsules (Lim and Sun, 1980); polyacrylate-anion-polyacrylate-cation-capsules (hydrogel) (Gharapetian *et al.*, 1986); polyacrylate-capsules (by coextrusion and interfacial precipitation (Sefton *et al.*, 1987)); negatively charged carboxymethyl-cellulose (CMC) – positively charged chitosan-capsules (Yoshioka *et al.*, 1990). All of them enable retention of cells in bioreactor systems allowing higher product titers and volumetric productivities due to higher cell densities. In addition, product retention, e.g. monoclonal antibodies produced by hybridomas, can be achieved by choosing the desired molecular weight cut-off of the membranes (e.g. Grdina and Jarvis, 1984: retention of mAb by alginate-PLL-polyvinylamine-capsules). Unfortunately, most of the aforementioned encapsulation processes are disadvantaged by the relative complexity of the encapsulation step itself which increases the costs of the process, and may be a source of contaminants. These drawbacks can be avoided when poly-

anion polycation encapsulation processes are used for the encapsulation of proteins, cell fragments, and hybridoma cells, using a solution of sodium cellulose sulfate (polyanion) and an aqueous solution of PDMDAAC (polycation) (Pommerening *et al.*, 1983; Merten and Dautzenberg, 1987), or using CMC and postively-charged chitosan (Yoshioka *et al.*, 1990). In order to test and optimize the method by Pommerening *et al.* (1983) for the encapsulation and subsequent cultivation of mammalian cells, in particular hybridoma cells, capsules were produced, whereby the following parameters were varied: first, the use of autoclaved or sterile-filtered sodium cellulose sulphate was compared with respect to cell growth and capsule characteristics. Second, three different samples of PDMDAAC were used, whereby the composition of the precipitation bath was varied (isotonic conditions: RPMI and PBS as base; versus hypotonic conditions: 0.1 × PBS and water). And third, the influence of different residence times (2 or 10 min) of the capsules in the precipitation bath on the cells and the capsule characteristics were tested. The results are discussed with respect to the advantages of the use of these capsules in an industrial process over other encapsulation systems (Lim and Sun, 1980; Gharapetian *et al.*, 1986; Sefton *et al.*, 1987).

Materials and methods

Hybridoma cell line

The cell line U0208 (supplied by H. Katinger, Vienna) was used. The cell line is a mouse × mouse hybridoma cell line and produces IgG. The cells were cultivated in DMEM (GIBCO), supplemented with 5% FCS (GIBCO) in Roux bottles (NUNC) at 36.5°C and 5% CO_2 humidified atmosphere. Every third to fourth day the cultures were split in a ratio of 1/3 to 1/5. The cells were tested for the absence of mycoplasma, according to the method of Chen (1977).

Encapsulation process

Cellulose sulphate. Sodium cellulose sulphate, prepared by a homogeneous process (Philipp and Wagenknecht, 1983) (degree of substitution: 0.46, viscosity: 209 mPa.s (1% solution)) was dissolved in aqua dest. in order to obtain a 3% solution. This solution was mixed with double-concentrated RPMI supplemented with 4% FCS in equal amounts and filtered using 0.2 µm Millipore membranes (GS/GV in a high pressure stainless filter holder, diameter 25 mm (XX45 025 00)).

A second solution of CS was prepared in the following way: the 3% solution of CS in aqua dest. was autoclaved (20 min, 121°C, 1 bar) and afterwards mixed with double-concentrated RPMI supplemented with 4% FCS in equal amounts.

Cell preparation. Cells were centrifuged (5 min, 1000 rpm) and were taken up in CS (1.5%)RPMI-2% FCS-solution for a final cell concentration of 2×10^5 cells per ml.

PDMDAAC. Three different samples of PDMDAAC were used: T32, MK10, MGV4/5, which were produced at different conditions of preparation. The molecular weights were 32 Kd for T32, 6 Kd for MK10 and 10 Kd for MGV4/5. The three samples were dissolved in aqua dest. to obtain a 4% solution. The solutions were autoclaved (20 min, 121°C, 1 bar) and diluted with either double-concentrated RPMI, or double-concentrated PBS, or 0.2 × PBS, or aqua dest. in equal amounts (final PDMDAAC concentration: 2%). Note: the viscosity of the precipitation bath has to be 10 to 100 times lower than that of the CS-droplets (Pommerening *et al.*, 1983).

Encapsulation. A CS-solution containing 2×10^5 cells per ml was pressed through a sterile syringe (approximate diameter 1 mm). The produced droplets fell (fall height: about 30 cm) into the agitated (40 rpm) precipitation bath (50 ml). The immediately-formed capsules were kept under agitation for 2 or 10 min. Afterwards the capsules settled out, the PDMDAAC-solution was aspirated, and the capsules were washed once in RPMI and once in DMEM-5% FCS. Per experiment, 1 ml capsules (which were between 50 and 60 capsules) were produced in this way.

Capsule cultures

One millilitre of capsules were cultivated in 9 ml DMEM-5% FCS in T-flasks (25 cm^2, NUNC: 1-63371). Each day, a sample of 1 ml of the supernatant was taken, the volume was replaced by an equal amount of fresh medium. The cultures were stopped after 13 days or 11 days in the cases of filtered-CS- or autoclaved-CS-based capsules, respectively.

The culture supernatant, which was free from capsules, was collected for testing the quantity of the IgG which was released from the capsules.

Determinations

The stability of the capsules was tested on day 0 and on day 5 or day 3 in the cases of filtered-CS- or autoclaved-CS-based capsules, respectively. The pressure which was necessary to crush a capsule was measured and expressed as p/capsule.

At the end of the cultures the cell number in the capsules and the cellular viability were determined, using a Thoma chamber and the trypan blue exclusion method. The liquid within the capsules was collected for testing the accumulated IgG.

The titration of mAb's in the supernatant and the liquid within the capsules was done according to Merten (1987).

Calculations

$$\text{Product retention} = \frac{\text{total IgG within the capsules}}{\text{total IgG in the culture}} \ (\mu g) \qquad (1)$$

$$\text{Specific production rate} = \frac{(ce - cb)\ (\ln xe - \ln xb)}{(t_e - t_b)\ (x_e - x_b)} \qquad (2)$$

expressed as pg IgG/cell × hour, where c_e is the total antibody concentration of the culture at the end of the culture, in pg, c_b is the total antibody concentration of the culture at the beginning of the culture, in pg, x_e is the total number of cells at the end of the culture, x_b is the total viable cell number at the beginning of the culture, and $t_e - t_b$ is the whole culture time in h.

Results

Preparation of the cellulose sulphate

Before the use of the CS for the encapsulation process, various lots were tested for cell toxicity (data not shown). The best one was used for all experiments. Using the same PDMDAAC-preparation, autoclaved CS versus filtered CS on cells and capsules were compared (Table 1). It is evident that capsules produced with autoclaved CS were inferior to those produced with filtered CS with respect to cell growth (e.g. 4.4×10^5 versus 0.35×10^5 cells per ml capsule volume for the system CS-MK10 after 13 days and 11 days in culture, respectively) and product accumulation (9.87 versus 1.05 µg IgG in total for the system CS-MK10). Similar results were obtained for the system CS-T32 and CS-MGV4/5. In addition, product retention was reduced when autoclaved CS was used. The retention was 70.4% and 38.2%, respectively, for the system CS-MK10, and 96.9% and 77.2%, respectively, for the system CS-MGV4/5. However, using the system CS-T32, no changes in product retention were observed. In some cases a reduced stability of the capsules was observed during the cultivation (T32 and MGV4/5 based capsules).

Influences of different PDMDAAC-samples and the composition of the precipitation bath on capsules and cells

In order to test the influence of the composition of the precipitation bath on the encapsulated cells and the characteristics of the capsules, two different PDMDAAC-samples (MK10 and T32) were diluted in four different solutions to obtain a 2% solution: two isotonic media: RPMI and PBS, and two hypotonic solutions were used: 0.1 × PBS and aqua dest. Although the hypotonic conditions

Table 1. Influences of the preparation of CS-solution (filtration versus autoclaving) on cell growth, cell viability, mAb-production, and capsule characteristics.

PDMDAAC CS	MK10[a]		T32[a]		MGV4/5[a]	
	fil[b]	aut[c]	fil[b]	aut[c]	fil[b]	aut[c]
Cell density (× 10^5/ml)	4.4	0.35	5.6	0.8	0.7	0.9
Viability (%)	25	20	50	33.3	33.3	42.9
Total IgG (µg)	9.87	1.05	18.57	2.05	0.90	0.26
IgG-retention (%)	70.4	38.2	99.4	100	96.9	77.2
Capsule characteristics	not changed		CS-aut: reduced stability		CS-aut: reduced stability	
p/capsule	6	13	25	4	75	2

[a]Cell inoculum 2 × 10^5 cells per ml capsule, starting viability: about 79%; precipitation bath composition: 0.1 × PBS; incubation time within the precipitation bath: 2 minutes.
[b]The CS-solution was filtered and prepared as described in Materials and methods, duration of the culture: 13 days.
[c]The CS-solution was autoclaved and prepared as described in Materials and methods, duration of the culture: 11 days.
[d]This test was done on day 5 for CS-fil, and on day 3 for CS-aut.

might be harmful for the cells, they were chosen due to their supporting effects on the capsule characteristics. The results, obtained using the system CS-MK10, are shown in Table 2.

It is evident that the different precipitation bath compositions had no influence on the clarity/turbidity of the capsules, and no effect on the long-term stability and capsule form. Their diam-

Table 2. Influence of the precipitation bath composition on cells and capsules when PDMDAAC-preparation MK10 was used[a].

Bath	RPMI	PBS	0.1 × PBS	Aqua dest.
Capsule-turbidity	+++	+++	+++	+++
Capsule diameter (mm)	2.4	2.8	2.1	2.4
Turbidity in the supernatant after some days	–	–	–	–
Deformed capsules	–	–	–	–
Sticking capsules	–	–	–	–
Stability: p/capsule	150–220 (d5:360)	95 (d5:260)	7–20 (reduced)	7–20 (reduced)
Total IgG (µg)	69.55	21.68	1.899	0.104
Product retention (%)	54.19	79.24	70.43	35.99
Viable cell count (× 10^5/ml)	26.4	21.6	4.4	0.4
Viability (%)	52.4	42.5	25	10
pg IgG/cell × hour	0.085	0.095	0.032	0.021

[a]The capsules were manufactured using a filtered 1.5% solution of CS and a 2% solution of PDMDAAC, the incubation of the capsules in the precipitation bath was 2 min. The cell specific data were obtained after a culture of 13 days.

Table 3. Influence of the precipitation bath composition on capsules when PDMDAAC-preparation T32 was used[a].

Bath	RPMI	PBS	0.1 × PBS	Aqua dest.
Capsule-turbidity	–	ND[b]	–	–
Capsule diameter (mm)	3.9	ND	3.7	ND
Turbidity in the supernatant after some days	+	ND	+	–
Deformed capsules	+	ND	+	–
Sticking capsules	++	ND	+/–	+/-
Stability: p/capsule	5–40	ND	3–33	20–40

[a]The capsules were manufactured using a filtered 1.5%. solution of CS and a 2% solution of PDMDAAC, the incubation of the capsules in the precipitation bath was 2 min. The cell specific data for RPMI and 0.1 × PBS are shown in Fig. 3.
[b]Not done.

eter ranged from 2.1 to 2.8 mm, which can be reduced easily by the use of a special droplet former (e.g. Hulst *et al.* 1985). The encapsulation process is easily done, and no sticking phenomena were observed. However, the mechanical strength of the capsules was reduced when hypotonic conditions were used during the encapsulation process: 7–20 p/capsule for hypotonic conditions versus 95–220 p/capsule with an increased stability during the culture for isotonic conditions. Unfortunately the capsules were completely opaque.

In the second part of Table 2 the cell specific data at day 13 after encapsulation are shown. It is evident that isotonic conditions supported cell growth and product accumulation. Cell cultures encapsulated using RPMI as basal medium of the precipitation bath showed a good cell density within the capsules after 13 days of culture (25.4 × 10^5 cells/ml capsule) and a total product accumulation of 69.5 µg IgG. Using PBS as basal medium for PDMDAAC-precipitation bath, more than 20 × 10^5 cells per ml were achieved. However, the total IgG-accumulation was only 21.7 µg. Both hypotonic precipitation bath conditions were disastrous for the cells. Little or no cell growth could be observed and IgG-accumulation was low.

The cell-specific productivity proves the superiority of the isotonic precipitation bath conditions in comparison with the hypotonic conditions. In all cases, the product retention was not 100%. It varied from 36% to 79% of the total produced IgG.

Similar experiments were carried out using PDMDAAC-sample T32 (Table 3). It is evident that the use of isotonic (RPMI) versus hypotonic precipitation bath conditions has a major influence on the capsule characteristics. Using isotonic conditions (RPMI or PBS (data not shown)) or 0.1 × PBS the integrity of the capsules was reduced. Five days after encapsulation, deformed capsules and membrane material were found in the culture supernatant. Sticking phenomena of the capsules (Fig. 1) originating from the separation process after encapsulation were observed in isotonic conditions. This phenomenon did not occur when using hypotonic encapsulation conditions. In general, the capsules, with an average diameter of 3–3.9 mm, were rather unstable (stability: 5-40 p/capsule), but completely clear (Fig. 1).

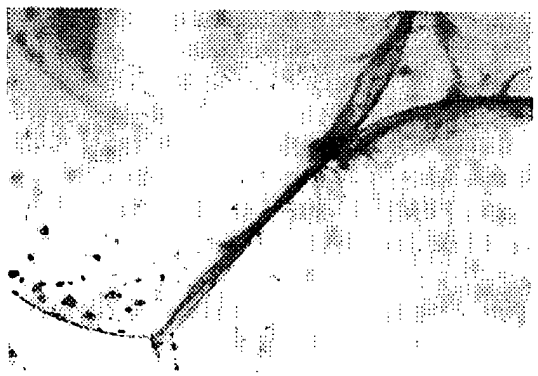


Fig. 1. Two 'sticky' capsules of the type CS (1.5%)-T32 (2%), precipitation bath: 0.1 × PBS, incubation time within the bath: 2 min. After a culture of 2 days, the developing clones within the capsules are visible.

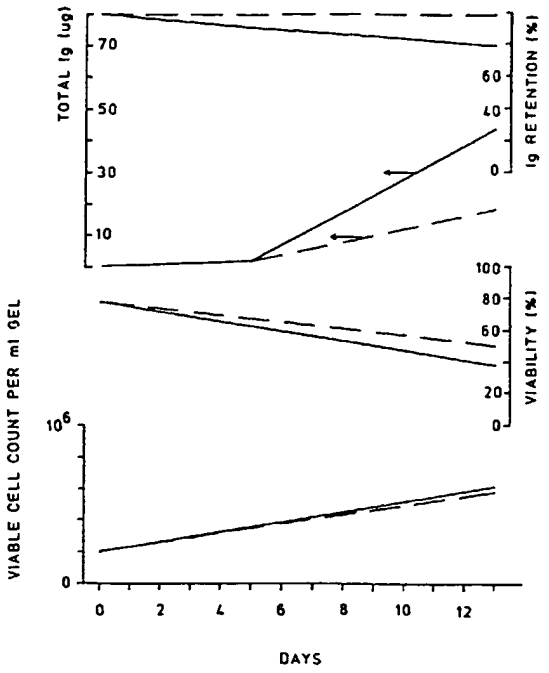


Fig. 2. Hybridoma culture within CS-PDMDAAC-capsules: (1) Capsule type: CS (1.5%)-T32 (2%), precipitation bath: RPMI, incubation time within the bath: 2 min: Full line; (2) Capsule type: CS (1.5%)-T32 (2%), precipitation bath: 0.1 × PBS, incubation time within the bath: 2 min: Broken line.

The comparison of RPMI and 0.1 × PBS as basal precipitation bath medium is shown in Fig. 2, with special respect to cell specific parameters. Using isotonic bath conditions, the cells produced more IgG and grew better than in capsules produced under hypotonic conditions. The cell viability in the RPMI-capsules was slightly inferior to that of the 0.1 × PBS-capsules 13 days after encapsulation. IgG-retention was only 80% in the case of RPMI-capsules and almost 100% for the 0.1 × PBS-capsules.

Influence of the incubation time of the capsules in the precipitation bath on the cells

The effect of incubation time of the capsules in the precipitation bath on the characteristics of the capsules and the cells was tested. CS-T32-capsules were produced, using RPMI and 0.1 × PBS as precipitation bath media. The residence time was chosen as 2 and 10 min, respectively. The results concerning the residence time of 2 min have been described (above) and are shown in Fig. 2. The effects of the 10 min incubation on the cells are significant (Fig. 3). Cell density and product concentration was higher when the capsules were incubated in an isotonic bath for 10 min compared to 2 min (about 26×10^5 cells/ml and 5.5×10^5 cells/ml, producing 44 and 51 µg IgG in total). Ten minutes of incubation obviously supports membrane formation, allowing a near 100% IgG-retention (only 80% in the capsule cultures, incubated for 2 min).

Contrarily, the residence time of 10 min had really disastrous effects on cell growth and product accumulation using hypotonic bath conditions, and is more unfavourable than a residence time of 2 min.

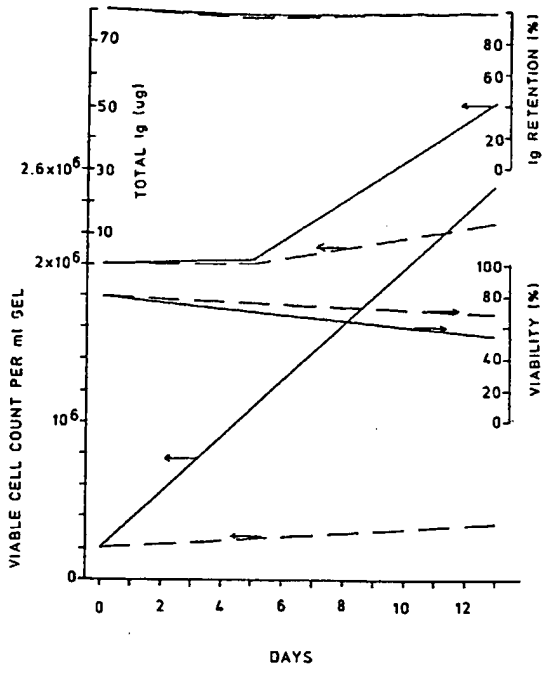
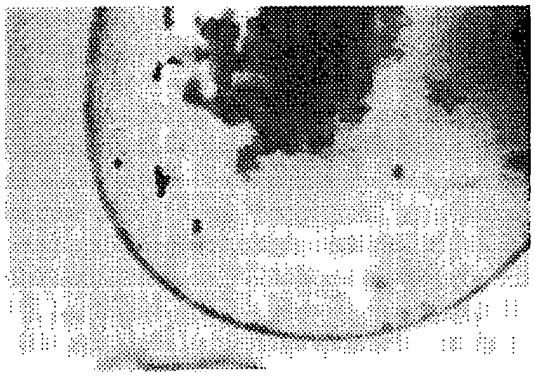
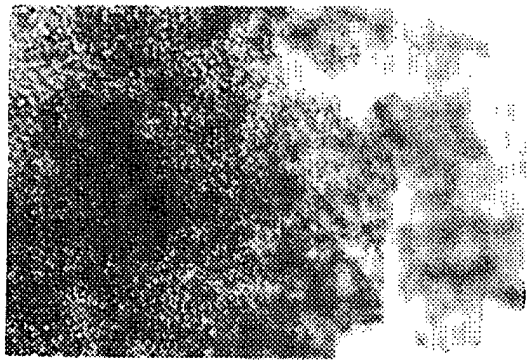


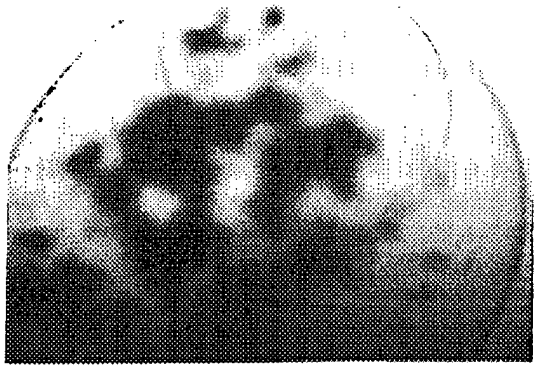
Fig. 3. Hybridoma culture within CS-PDMDAAC-capsules: (1) Capsule type: CS (1.5%)-T32 (2%), precipitation bath: RPMI, incubation time within the bath: 10 min: Full line; (2) Capsule type: CS (1.5%)-T32 (2%), precipitation bath: 0.1 × PBS, incubation time within the bath: 10 min: Broken line.



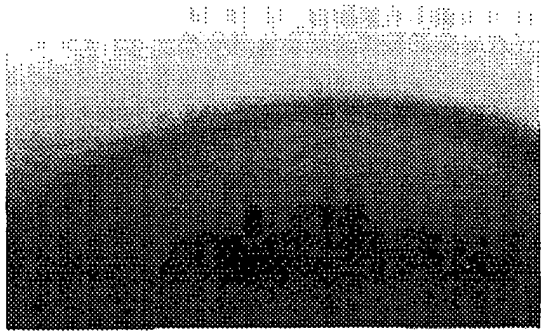
A



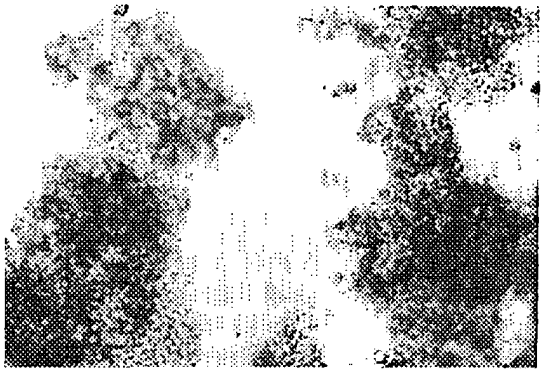
B



C



D



E

Fig. 4. Hybridoma culture within CS-PDMDAAC-capsules, the photos were taken from the culture shown in Fig. 3, experiment 1: (a) Capsules and cells after a culture time of 5 days; (b) Cells within a capsule after a culture time of 5 days; (c) Capsules and cells after a culture time of 12 days; (d) Cells within a capsule after a culture time of 12 days; (e) Capsules and cells after a culture time of 13 days.

Finally, to give some impressions about cellular proliferation within the capsules, Fig. 4a,b shows capsules and encapsulated cells after 5 days in culture, Fig. 4c,d shows capsules and encapsulated cells after 12 days in culture, and Fig. 4e a capsule and cells 13 days after encapsulation. The photos were taken from the cultures of capsules, which were produced with CS-T32 in RPMI using a residence time of 10 min.

Discussion

It has been demonstrated that the encapsulation process using sodium cellulose sulphate as polyanion and PDMDAAC as polycation, is a suitable tool for the cultivation of mammalian cells at high densities.

The variation of the encapsulation parameters had significant influences on the capsule characteristics and the cell cultures within the capsules. Although the use of autoclaved CS would facilitate the sterilization process, our CS-preparation showed really inferior results when it was used compared to the filtered form. The cells grew more slowly and product accumulation was retarded. In the systems CS-MK10 and CS-MGV4/5 the IgG-retention was decreased in comparison to capsules manufactured using filtered CS. Both autoclaved CS-T32 and CS-MGV4/5 based capsules showed a reduced stability when compared to filtered CS based capsules. These results show that the properties of CS during autoclaving can drastically change, thus remarkably deteriorating the properties of the capsules. In future, attention has to be paid to developing types of CS that do not lose their original properties during autoclaving. This is of high interest due to the problems arising when a 1.5% CS-solution (viscosity of a 1% solution: 209 mPa.s) has to be filtered.

During the study, three different PDMDAAC-samples (MK10, T32, MGV4/5) using three different conditions of preparation were used. In all cases, four different precipitation bath compositions were employed – two isotonic (RPMI, PBS) and two hypotonic (0.1 × PBS, aqua dest.). It became clear that the use of isotonic precipitation bath conditions was, in all cases, better for cell growth and Ig-production, when compared to hypotonic conditions. However, the effects of the precipitation bath compositions on the capsule characteristics were quite different. In the case of T32 and MGV4/5 (data not shown), the use of aqua dest., and 0.1 × PBS to some extent, increased the long-term stability of the capsules. That meant that almost no membranous material could be found in the culture supernatant. The ease of the encapsulation process, when hypotonic conditions were used, was striking. No clumping or sticking effects were observed. The use of isotonic conditions (RPMI, PBS) produced rather 'sticky' capsules. The other capsule characteristics, like stability or turbidity/clarity of the capsules, were not affected by the osmolarity of the precipitation bath. Only MK10-based capsules showed a reduced stability when hypotonic encapsulation conditions were employed, other capsule characteristics were not affected by the different osmolarities of the encapsulation bath.

The residence time of 10 min of the capsules in the precipitation bath improved the capsule characteristics in comparison with 2 min; isotonic conditions were favourable for the cells; in contrast, hypotonic conditions were disastrous for cell culture.

One aspect is very interesting. If it is necessary or desirable for the production process, it is possible to choose between a complete product (IgG and IgM (data not shown)) retention (CS-T32 system) or a product releasing capsule system (MK10, MGV4/5). The product releasing system is easily realizable for IgG, however, IgM is mostly retained due to the higher molecular weight (retention: 94–100%, data not shown).

The DAMON process also allows the choice of a product retention or release (Jarvis *et al.*, 1983; Grdina and Jarvis, 1984). Recently, King *et al.* (1987) reported an optimized process, by which the membrane molecular weight cut-off could be controlled by the viscosity of PLL and the reaction time between alginate and PLL. Gharapetian *et al.* (1987) were able to influence the capsule characteristics (membrane formation, capsule strength, expansion in physiological saline, product retention) by the comonomer feed composition and the molar ratio of monomer to initiator. The encapsulation method of Yoshioka *et al.* (1990) allows a complete retention of IgG, however, it is unknown whether the capsule properties are influenced by the encapsulation conditions.

In one case (CS-MK10), the capsules were very turbid and no visual cell control was possible. This can be a problem, but might be solved by using a better fitting PDMDAAC-sample.

The actual diameter of the capsules was too large. However, it can be decreased very easily to values of 150–500 μm, which is an acceptable range for the sufficient diffusion of oxygen, nutrients, and wastes (Glacken *et al.*, 1983), when droplet formers are used (Hulst *et al.*, 1985).

Comparing this encapsulation system with the DAMON process (Lim and Sun, 1980) or the two

polyacrylate based capsule systems (Gharapetian *et al.*, 1986; Sefton *et al.*, 1987), it is evident that this new process has some advantages. The encapsulation and separation steps are very fast and simple process steps, whereas a lot of additional process steps have to be performed with the other systems. The DAMON system is based on the formation of alginate beads, to which PLL has to be added. Then a hardening process is necessary (e.g. by the use of PEI). Finally, the alginate has to be solubilized before cultivation can be started. Separation and washing are necessary between each step. Similar problems arise when the method of Gharapetian *et al.* (1986) is used. After the first precipitation bath, two additional incubation baths with intermittent washing and separation steps are necessary in order to improve the capsule characteristics and perhaps stability. Only the polyacrylate process of Sefton *et al.* (1987) seems to be a rather simple one because it avoids too many washing and separation steps. The process described by Yoshioka *et al.* (1990) appears to have the same advantages as the encapsulation process presented in this paper.

The encapsulation system based on the membrane formation between CS and PDMDAAC can be used for the culture of mammalian cells. The comparison of this process with others showed that the new system has some advantages over most of the other systems, mainly with respect to the easiness of the process.

The system-specific problems were shown and it is evident that this system has to be further optimized with special reference to autoclavable CS, to an increase in the stability of the capsules, to avoiding sticking problems in some cases, and for tailoring the desired molecular weight cut-off of the capsule membranes, before it can be used as an industrial process.

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